centration of total amino acids is greater in serum than in the comparable plasma(18). This same relationship has been found to hold for individual amino acids(19).

Summary. Normal premature infants fed a diet deficient in phenylalanine developed changes in their bone marrow within 24 to 48 hours. These marrow changes consisted specifically in cytoplasmic vacuolization of erythroid precursors. Vacuoles also occurred in the myeloid elements. The vacuoles of the red cell precursors were associated with marked depression of serum phenylalanine concentrations to levels of 4 and 6 micromoles per liter. These effects were reversible within 48 hours by the addition of 100 mg per kg of phenylalanine to the diet. Any possible effects of the phenylalanine deficiency on other amino acids was obscured by the increased serum and urine concentrations of these amino acids resulting from increased intake provided by the proprietary casein hydrolysate preparation. Addition of valine, alanine, and methionine to the normal and deficient diets did not produce marrow changes or other toxic effects.

The authors gratefully acknowledge their indebtedness to Miss Meta Bohanan, R.N., and her staff for help in carrying out the observations on the patients and to Drs. H. P. Baker, R. Feigin, F. K. Kennedy, D. Palant, N. Pleva and P. Sexton of the resident staff for major contributions to the care and study of the patients.

1. Sherman, J. D., Greenfield, J. B., Ingall, D., New Eng. J. Med., 1964, v270, 810.

2. McCurdy, P. R., J.A.M.A., 1961, v176, 588.

3. Rosenbach, L. M., Caviles, A. P., Mitus, W J., New Eng. J. Med., 1960, v263, 724.

4. Saidi, P., Wallerstein, R. O., Clin. Research, 1960, v8, 131.

5. Saidi, P., Wallerstein, R. O., Aggeler, P. M., J. Lab. & Clin. Med., 1961, v57, 247.

6. McCurdy, P. R., Pierce, L. E., Rath, C. E., New Eng. J. Med., 1962, v266, 505.

7. Di Guglielmo, L., Le malattie eritremiche ed eritroleucemiche, Rome, Il pensiero scientifico editore, 1962, 110.

8. Holt, L. E., Jr., 30th Ross Pediatric Research Conference, Ross Laboratories, Columbus, Ohio, 1958, 11.

9. Toyston, N. J. W., Parry, T. E., Arch. Dis. Child., 1962, v37, 430.

10. Spackman, D. H., Stein, W. H., Moore, S., Anal. Chem., 1958, v30, 1190.

11. Moore, S., Stein, W. H., J. Biol. Chem., 1954, v211, 907.

12. Smith, I., Chromatographic and Electrophoretic Techniques, William Heineman, London, 1960, v1, 83.

13. Morrison, R. I., Biochem. J., 1953, v53, 474.

14. Peters, J. H., J. Biol. Chem., 1942, v146, 179.

15. Hirs, C. H. W., Stein, W. H., Moore, S, ibid., 1954, v211, 941.

16. Ponder, E., Ponder, R. V. O., J. Lab. and Clin. Med., 1942, v28, 316.

17. Ingall, D., Sherman, J. D., Cockburn, F., Klein, R., 1964, unpublished.

18. MacFayden, D. A., J. Biol. Chem., 1942, v145, 387.

19. Stein, W. H., Moore, S., ibid., 1954, v211, 921.

Received September 2, 1964. P.S.E.B.M., 1965, v118.

Rapidly Labeled Rat Liver Nuclear RNA Particles.* (29809)

KENNETH S. MCCARTY, JOHN LASZLO† AND JOHN R. OVERMAN

Departments of Biochemistry and of Medicine, Duke University Medical Center, Durham, N. C.

The mechanism of nuclear RNA synthesis in regenerating rat liver involves the production of a rapidly labeled RNA copy of DNA. Initial investigations from this laboratory indicate that pulse labeled nuclear RNA could be isolated, purified and sedimented as a clear pellet in the presence of Mg^{++} ions. This pellet contained 60-100% of the radioactivity. Experiments concerning the nature of this pellet comprise the basis of this report.

Materials and methods. 1. Preparation of rat liver nuclei: Wistar rats, 150-250 g, were

* This research was supported in part by USPHS Grants GM-06975-03, HD-0068-06, 2 G-516, Nat. Science Foundation G-13194 and Am. Cancer Soc. Grant A.C.P363.

† Supported in part by USPHS Student Training Grant CSP-8520-C2.

hepatectomized (20-40%) and immediately injected with 10 μc of orotic acid 6 C^{14} (specific activity 3.45 mc/mM, New England Nuclear Corp.) 90 minutes prior to sacrifice. The livers were removed, quickly frozen on dry ice, and homogenized in a Potter Elvehjem homogenizer (6-8 strokes at 600 rpm) in 5 volumes of 5% sucrose containing .04 M citric acid. All solutions were maintained at 4°C. The homogenate was diluted 4-fold with homogenizing solution and passed through an 80 mesh stainless steel cloth held in a millipore filter adaptor. Nuclei were concentrated by centrifugation at 400 $\times$ g for 10 minutes in conical centrifuge tubes and washed three times in 5% sucrose containing .04 M citric acid. Nuclei were resuspended by light homogenization in 2.0 M sucrose containing .04 M citric acid and then centrifuged in a swinging bucket rotor (SW 25.1) at 90,000 $\times$ g for 90 minutes in a Spinco model L preparative ultracentrifuge. The supernatant sucrose was decanted and the tubes permitted to drain to provide a clean pellet of nuclei. The nuclei were examined in the electron microscope to determine the degree of purification.

2. Preparation of nuclear RNA: Twenty mg of Mg^{++} bentonite(1) were added to nuclei obtained from 10 g of hepatectomized liver. The nuclei were gently homogenized in 40 ml (10 g of initial liver) of a solution containing .03 M Tris·HCl (pH 7.5), .01 M $MgCl_2$, 0.5% sodium lauryl sulfate and 0.1% trimethylamine. The homogenate was heated to 60°C and an equal volume of preheated saturated phenol (70 cc of 88% liquid phenol and 30 cc of homogenizing solution) was added. The mixture was stirred vigorously at 60°C for 5 minutes and then plunged into a dry ice-alcohol slurry. The heating, mixing and cooling procedure was repeated twice. The solution was centrifuged at 30,000 $\times$ g for 10 minutes and the aqueous phase carefully removed and ether extracted to remove the phenol. RNA was precipitated overnight by adjusting the aqueous phase to 2% K acetate and with 2.5 volumes of ethanol. The RNA precipitate was washed twice with cold 67% ethanol.

3. Preparation of cytoplasmic RNA: Cytoplasmic RNA was prepared from the supernatant of centrifuged liver homogenate. The supernatant was adjusted to 0.5% sodium lauryl sulfate, 0.1% trimethylamine, .03 M Tris pH 7.5, .01 M $MgCl_2$ and 0.1% bentonite. The solution was gently homogenized, brought to 60°C, phenol extracted and alcohol precipitated as described above.

For RNA preparations free of DNA, an additional DNA'se step (2.5 μg/ml deoxyribonuclease) was included. This was followed by bentonite, phenol, ether and alcohol precipitation as described above.

Commercial yeast RNA (Worthington) was also purified as described above to remove traces of ribonuclease activity.

4. Preparation of nuclei, nuclear and cytoplasmic RNA for electron microscopy: Samples were fixed for 30 minutes in 2% veronal buffered osmium tetroxide, pH 7.4. The fixed samples were dehydrated in alcohol and embedded in araldite. Sections were cut with a Porter-Blum microtome and stained with uranyl acetate for one hour. Micrographs were made with an RCA-EMU-3F double condenser electron microscope.

5. Sucrose density gradients: Linear sucrose density gradients 5-20% were prepared according to Martin and Ames(2) using a Technicon proportioning pump to prepare 4 replicate tubes. A linear gradient was obtained by selecting conditions to remove fluid from the mixing flask at twice the rate of addition of the 5% sucrose. Linearity was checked by determining the refractive index of the extra gradient tube.

6. Analytic methods: RNA was determined by the orcinol procedure(3), and DNA was determined by the p-nitrophenylhydrazine procedure(4). Base analysis was followed by column, paper and thin layer chromatography. Protein was analyzed by the method of Lowry(5). Protein contaminants were assayed by an amino acid analysis(6) after 6 N HCl digestion for 18 hours. Radioactive analyses were performed using a Tri Carb scintillation counter. Bray's solution(7) was used as the scintillator.

Results. Sucrose isolated nuclei were examined in the electron microscope for their purity (Fig. 1a).

When purified rat liver nuclear nucleic acids were dissolved in 11.5 ml of .03 M Tris HCl pH 7.5, .01 M $MgCl_2$ and subjected to 105,000

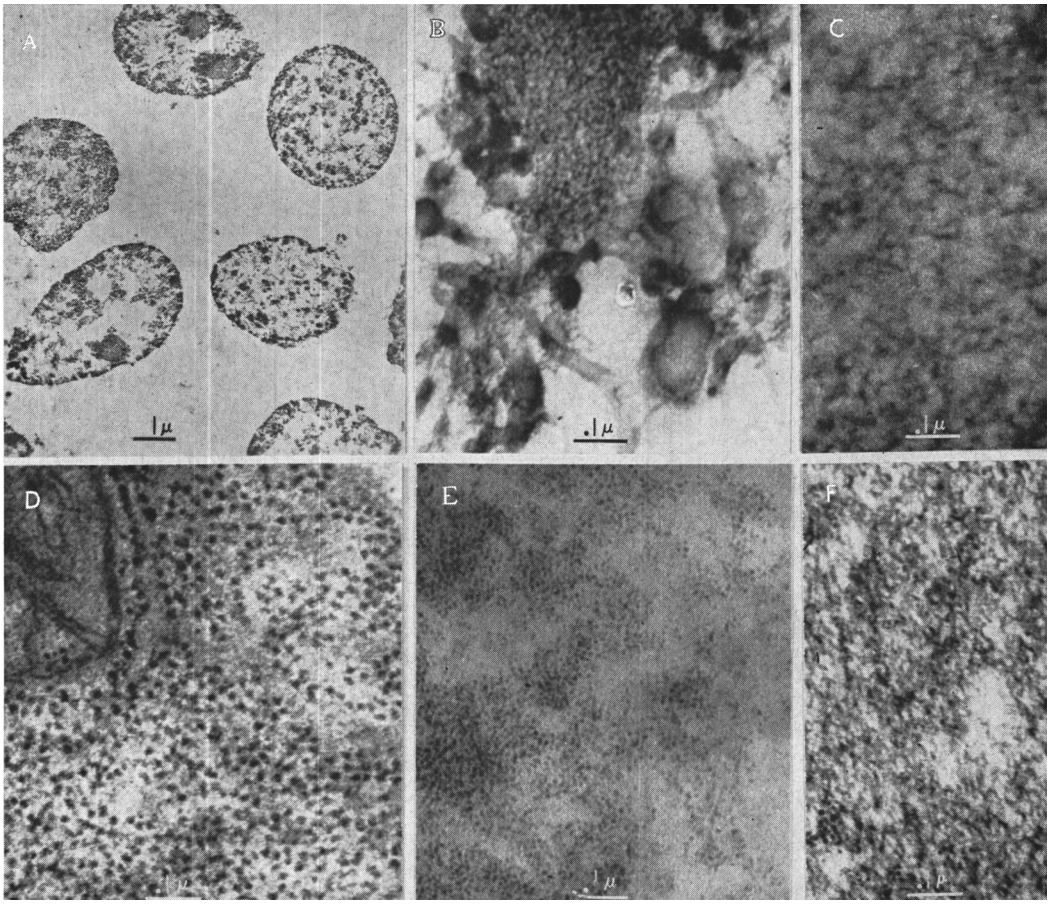

FIG. 1. Electron micrographs of liver nuclei and RNA pellets. A—Sucrose isolated nuclei × 3120; B—nuclear RNA-DNA × 72000; C—enlarged section of an intact, isolated nucleus × 72,000; D—intact liver cytoplasm × 72,000; E—rat liver cytoplasmic RNA × 72,000; F—yeast RNA × 72,000.

× *g* for 2 hours a perfectly clear translucent pellet was obtained. Electron microscopy of this pellet revealed small electron dense particles organized singly or in clusters along interlacing membranous structures (Fig. 1b). It was found that these structures resembled some of the cytological characteristics seen in the intact isolated nucleus (Fig. 1c). That the interlacing membranous structures were composed of DNA was suggested by the loss of these membranes following deoxyribonuclease treatment (2.5 μg/ml), leaving only the small dense particles. Furthermore, in nucleic acid preparations treated with ribonuclease (.1 μg/ml), the small dense particles were removed leaving only the membranous structures intact.

The ultrastructure of liver cytoplasm is shown in Fig. 1d. Purified Mg^{++} aggregated RNA yielded electron dense particles (Fig. 1e). Similar particles also were observed from commercial yeast RNA (Fig. 1f). The spectrum of both nuclear and cytoplasmic RNA revealed a maximum at 260 mμ, a minimum at 230 and 260/280 ratio of 2.0.

The chemical composition of the deoxyribonuclease-treated nuclear RNA was checked for purity and there was no detectable DNA, as determined by the p-nitrophenylhydrazine reaction or by thin layer chromatography of the hydrolyzed bases. Protein could not be detected by the Lowry method. The RNA was therefore examined for hydrolyzed protein by a ninhydrin amino acid analysis. This

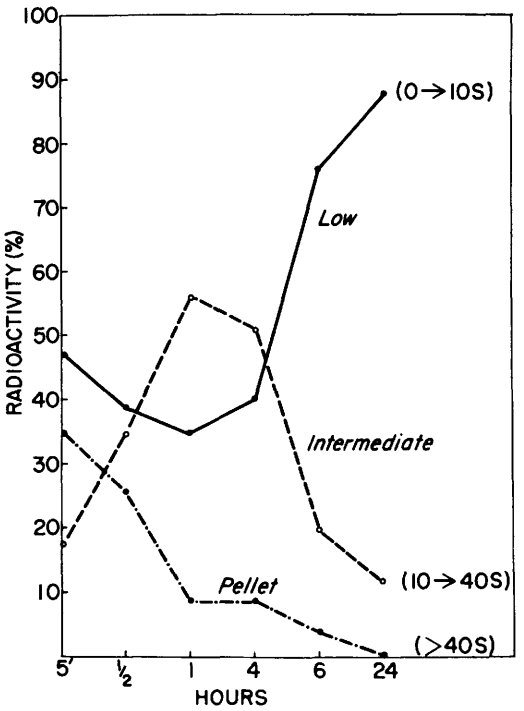

FIG. 2. Distribution of pulse nuclear RNA. Relative distribution of radioactivity of pooled fractions collected from a 5-20% sucrose layered over 2 M sucrose. Samples were fractionated as described in text. 10 μc of Na^+ Orotate 6 C^{14} was injected immediately following partial hepatectomy. Duration between time of injection and sacrifice varied from 5 min to 24 hr.

analysis revealed the presence of glycine and ammonia as the major contaminants most probably from the degradation of the RNA bases. Other amino acids were present in negligible quantities, to account for less than 1% protein contamination. In order to exclude the possibility of the electron dense structures representing phospholipid, an additional chloroform-methanol (3:1) treatment of the RNA pellet was performed. The electron dense particles survived this extraction.

The presence of Mg^{++} was essential for formation of a pellet of RNA sedimenting at 105,000 $\times$ g for 90 minutes. This was shown by the fact that removal of Mg^{++} by addition of EDTA followed by fractionation on a column of Sephadex G-25, prevented the sedimentation. The Mg^{++} free RNA, either as the K^+ or NH_4^+ salt could be reassociated by addition of .010 M Mg^{++}.

An outline of one RNA pulse labeled experiment is given in Table I. It should be noted that most of the radioactive alcohol-precipitable RNA was centrifuged down with the Mg^{++} associated RNA pellet.

Fig. 2 shows a time course experiment by procedures modified after those of Hiatt(9). In these experiments RNA was isolated in the presence of .001 M $MgCl_2$. The nuclear RNA fractions were divided into 3 parts after centrifugation in 5-20% sucrose density gradient. Tubes 20-30 were pooled and designated as the light fraction (0 to 10s), tubes 10-20 were pooled and designated intermediate fraction 10 to 40s. A pellet (> 40s) was resuspended from the bottom of the tube and added to the remaining 10 tubes and designated as the heavy fraction. It may be noted that a large percentage of the label is present in the pellet fraction at 5 minutes after orotic acid C^{14} was injected intraperitoneally. There was a progressive loss of relative incorporation from the pellet into the intermediate fraction during the course of 6 hours. Thereafter the intermediate fraction lost its share of radioactive label to the lower molecular weight fraction.

Discussion. The evidence presented here demonstrates that in the presence of .01 M Mg^{++}, RNA particles (aggregates?) may be observed from protein free preparations of nuclear, cytoplasmic or yeast RNA. In addition to these particles, the nuclear RNA preparations contaminated with DNA contain interlacing membranous structures. Allfrey *et al* have shown remarkably similar features in intact trypsinized calf thymus nuclei(10). The evidence that the membranous components are DNA is based on the fact that they

TABLE I. Sedimentation of Pulse Labeled Nuclear RNA.

Initial RNA before centrifugation (cpm)	Solvent* system	Pellet (cpm)	Supernatant (cpm)
38,000	.03M K* acetate pH 7.5 .01M $MgCl_2$	35,000	0
30,800	.03M K* acetate pH 7.5 .1 M K* EDTA	0	32,000

* Pulse labeled nuclear RNA No. 57, was centrifuged at 105,000 $\times$ g for 2 hours.

are absent after deoxyribonuclease attack, even though they survived vigorous homogenization in sodium lauryl sulfate, trimethylamine, hot phenol extraction, freezing, thawing, alcohol precipitation, chloroform-methanol (3:1) and ether extraction. Preparations of DNA treated with ribonuclease show only membranous structures without RNA particles(8).

It is most probable that the RNA particles represent an interaction between isolated RNA and a divalent ion as Mg^{++}. This interaction may be analogous to that observed with synthetic polynucleotides(11). These observations would imply that the electron density of ribosomes is primarily attributable to their RNA content and is not dependent on nucleoprotein.

The precipitability of nuclear RNA is dependent upon the endogenous Mg^{++} concentration of the isolation medium as well as the Mg^{++} concentration in the sucrose density gradient. At low Mg^{++} concentrations (.001 M Mg^{++} in the isolation medium and none in the sucrose density gradient) the per cent of radioactivity is distributed in the fractions as a function of time. Within 5 minutes after an orotic acid C^{14} label, a large portion of the radioactivity is associated with the pellet (Fig. 2). The apparent progressive loss of radioactivity from the pellet into the intermediate and then to low molecular weight fractions suggests that the earliest labeled RNA is the highest molecular weight and that the later appearing fractions represent progressively disaggregated forms. These results are similar to those reported for HeLa cells by Scherrer and Darnell(12). In addition to the ribosomal and transfer RNA peaks as seen by optical density measurements, 2 heterogeneous radioactive fractions were observed. One fraction sediments between 30-45 S and a second fraction > 45 S results in a high specific activity pellet. The amino acid incorporation capacity, Mg^{++}/P ratios, and sedimentation characteristics in the presence of divalent cations is to be reported elsewhere (8). Although these data are consistent with the hypothesis that the most rapidly labeled RNA is a high molecular weight component synthesized in the nucleus which is then either dissociated to smaller components or transported to the cytoplasm, we feel that a direct translation into molecular weight may be misleading unless the effect of that particular Mg^{++} concentration has been predetermined.

The authors are grateul for helpful suggestions made by Drs. Walter Guild, Robert Bonar, Montrose Moses, and William Lynn. We wish to acknowledge the careful technical assistance given by Mr. Clarence Ellis, Mr. Ronald Senigen and Mr. Oliver Brown.

1. Petermann, M. L., Pavlovec, A., J. Biol. Chem., 1963, v238, 3717.

2. Martin, R. G., Ames, B. N., ibid., 1961, v236, 1372.

3. Ceriotti, G., ibid., 1955, v214, 59.

4. Webb, J. M., Levy, H. B., ibid., 1953, v213, 107.

5. Lowry, O. H., Rosebaugh, N., Farr, A. L., Randall, R. J., ibid., 1951, v193, 265.

6. McCarty, K. S., Exp. Cell Res., 1962, v27, 230.

7. Bray, G. A., Analyt. Biochem., 1960, v1, 279.

8. McCarty, K. S., Laszlo, J., in preparation.

9. Hiatt, H. H., J. Molec. Biol., 1962, v5, 217.

10. Allfrey, V. G., Littau, V. C., Mirsky, A. E., Proc. Nat. Acad. Sci., 1963, v49, 414.

11. Eisinger, J., Faway-Estrup, F., Shulman, R. G., Biochim. Biophys. Acta, 1963, v72, 120.

12. Scherrer, K., Darnell, J. E., Biochem. Biophys. Res. Commun., 1962, v7, 486.

Received September 2, 1964. P.S.E.B.M., 1965, v118.